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13. ABSTRACT ***(Maximum 200 Words)***
The cell surface glycoprotein, EMMPRIN, enriched on the surface of tumor cells, stimulates production f several MMPs by stromal cells and by the tumor cells themselves, implying that EMMPRIN is an important regulator of MMP production during tumorigenesis *in vivo*, making it a potentially crucial component of tumor progression. We examined whether increased expression of EMMPRIN in weakly-malignant MDA-MB-436 and MCF-7 mammary carcinoma cells and in non-malignant MCF10A mammary epithelial cells and MDCK canine kidney epithelial cells induces anchorage independent-growth and invasion, hallmark characteristics of the malignant phenotype. Using a soft agar assay to assess anchorage-independent growth, increased expression of EMMPRIN stimulated 10-fold and 5-fold increases respectively in the number of colonies formed by MDA-MB-436 and MCF-7 cells. When assessed by invasion of reconstituted basement membranes, we found that EMMPRIN enhanced invasiveness 2 to 9-forld in MDA-MB-436, MCF-7, MCF10A and MDCK cells. Next, we examined changes in MMP production. Increased expression of EMMPRIN in MDA-MB-436, MCF-7 and MCF10A cells yielded a 2-fold increase in MMP-2 assayed by gelatin zymography. By Western analysis we saw a 2-fold increase in MT1-MMP, an enzyme involved in the activation of MMP-2 and in tumor cell invasion. Hyaluronan (HA), a component of tumor cell pericellular matrix has also been shown to promote tumor progression. Using a competitive ELISA-like method, we found that increase expression of EMMPRIN in MDA-MB-436 and MDCK cells gave rise to HA levels 2-fold greater than controls. Using HA oligomers to compete with endogenous HA polymer, we reversed EMMPRIN-enhanced colony formation of MDA-MB-436 and MCF-7 cells to near control levels. These data imply that EMMPRIN promotes tumor progression, most likely through stimulation of HA and MMPs. Therefore,antagonizing EMMPRIN may be a useful therapeutic avenue towards slowing tumor progression.

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Introduction

A critical step in tumorigenesis is proteolytic modification of the peri-cellular matrix surrounding tumor cells by matrix metalloproteinases (MMPs). Stromal cells associated with tumors are responsible for the production of most tumor MMPs. Studies from our laboratory and those of our collaborators have shown that EMMPRIN (extracellular matrix metalloproteinase inducer), a tumor cell surface glycoprotein, stimulates the production of several MMPs by fibroblasts and endothelial cells. Antisense cDNA and ribozyme constructs were utilized in an attempt to inhibit EMMPRIN expression in TA3/ST cells, a murine breast carcinoma cell line. These constructs were not efficient in blocking EMMPRIN expression and consequently, were inactive *in vivo*. However, transfection and injection experiments done in collaboration with Dr. Stanley Zucker have shown that MDA-MB-436 human breast cancer cells transfected with GFP-EMMPRIN produce much larger tumors in nude mice than vector-transfected cells. These same EMMPRIN-transfected cells become more invasive than their control counterparts both *in vivo* and in a Boyden chamber invasion assay and they exhibit enhanced levels of MMP-2. To confirm and extend this work, we examined whether increased expression of EMMPRIN in weakly-malignant MDA-MB-436 and MCF-7 mammary carcinoma cells and in non-malignant MCF10A mammary epithelial cells and MDCK canine kidney epithelial cells induces anchorage-independent growth and invasion, hallmark characteristics of the malignant phenotype and what mechanism underlies these malignant changes.

Body

Task 1: To show that inhibition of EMMPRIN protein expression leads to a reduction in breast tumor growth.

In an effort to show that inhibition of EMMPRIN protein expression leads to a reduction in breast tumor growth and metastasis, we constructed both human and murine EMMPRIN antisense cDNAs and ribozymes. These constructs were then transfected into TA3/ST murine breast carcinoma cells or human MDA-MB-231 human breast carcinoma cells. The stably transfected cells were then injected into the tail vein of syngeneic mice to see if growth and metastasis of the tumor would now be reduced. Neither of the constructs was efficient in blocking EMMPRIN expression, and consequently were inactive *in vivo.*

Task 2: To demonstrate a rise in MMP production by microvascular endothelial cells as well as umbilical vein endothelial cells in response to EMMPRIN treatment.

Tumor angiogenesis plays an important role in cancer progression (1). Since up-regulation of MMP activity is required for angiogenesis and tumor endothelial cells express MMPs (2,3), Dr. Stanley Zucker, in collaboration with my mentor's laboratory examined whether EMMPRIN stimulates MMP production by human endothelial cells, as well as fibroblasts. In three separate experiments, purified EMMPRIN was shown to cause a significant stimulation of stromelysin-1, gelatinase A and collagenase (MMP-1) production (but not TIMP-1 or TIMP-2) in human umbilical vein endothelial cells. The effect was most marked for stromelysin since the background levels produced by the untreated endothelial cells were low. In contrast, VEGF, an established mediator of angiogenesis, had little effect on stromelysin or gelatinase A and a relatively strong effect on MMP-1; but, the most dramatic effect of VEGF was on TIMP-1 stimulation (4). These effects were not due to influences on proliferation. Thus, EMMPRIN is a more potent inducer of MMP synthesis than VEGF.

Further evidence for a role for EMMPRIN in tumor angiogenesis has developed out of the collaboration between Dr. Zucker's and my mentor's laboratories. A line of MDA-MB-436 human breast cancer cells that grows slowly *in vivo* was transfected with EMMPRIN cDNA or with vector alone, and tested for tumor growth and metastasis in nude mice. Whereas control vector transfectants formed very small, non-metastatic tumors, the EMMPRIN transfectants in most cases formed large palpable tumors and metastasized to numerous sites. The latter tumors, but not the former, were found to be highly vascularized.

My major role in these projects has been to develop a better system for demonstrating EMMPRIN-dependent MMP stimulation in endothelial cells. I examined MMP production by human umbilical vein endothelial cells that have been infected with a recombinant, full-length EMMPRIN adenovirus. This approach was utilized due to the availability of adenoviral reagents in the laboratory as well as ease of amplification and purification.

In the past, we have used EMMPRIN purified from LX-1 cells, a human lung carcinoma cell line. The purification process is tedious, time-consuming, labor-intensive and yields small quantities of EMMPRIN. Utilizing the adenoviral approach not only provides us with another way to generate EMMPRIN, but also eliminates possible contaminants that may be in our EMMPRIN preparations purified from LX-1 cells. This approach has been shown to provide more reproducible and efficient stimulation of MMP production by fibroblasts.

In our adenoviral approach, human umbilical vein endothelial cells were infected with the EMMPRIN adenovirus. We expected that EMMPRIN would be produced by the endothelial cells and bind to its putative receptor on neighboring endothelial cells, leading to a series of signaling events that would ultimately result in MMP production. This approach failed to produce any repeatable results.

However, this methodology has led to a confirmation and extension of a homotypic interaction involving EMMPRIN on tumor cells. As previously stated, stromal cells associated with tumors are responsible for the production of most tumor MMPs. However, recent studies from our laboratory and those of our collaborators have shown that EMMPRIN not only stimulates the production of several MMPs by fibroblasts and endothelial cells, but also by the tumor cells themselves. Previous experiments in collaboration with the laboratory of Dr. Zucker have shown that MDA-MB-436 mammary carcinoma cells transfected with EMMPRIN produce much larger tumors in nude mice than vector-transfected cells. These same EMMPRIN-transfected cells become more invasive than their control counterparts both *in vivo* and in a Boyden chamber invasion assay and they exhibit enhanced levels of MMP-2 (5).

To confirm this invasive phenotype and extend this phenomenon to non-cancerous cells, we have employed a Matrigel invasion chamber assay. MDA-MB-436 (Fig. 1A), MCF-7 (mammary carcinoma) (Fig. 1B), MCF10A (mammary epithelial) (Fig. 1C) and MDCK (canine kidney epithelial) cells (Fig. 1D) were either transfected with EMMPRIN or infected with a full-length EMMPRIN adenovirus and then placed in the Matrigel chambers for 24 hours. The number of cells that invaded were then counted and compared with controls. In each case, elevated EMMPRIN expression corresponded to increased invasiveness over controls with an MMP2/9 inhibitor capable of reducing EMMPRIN-enhanced invasion to near control levels.

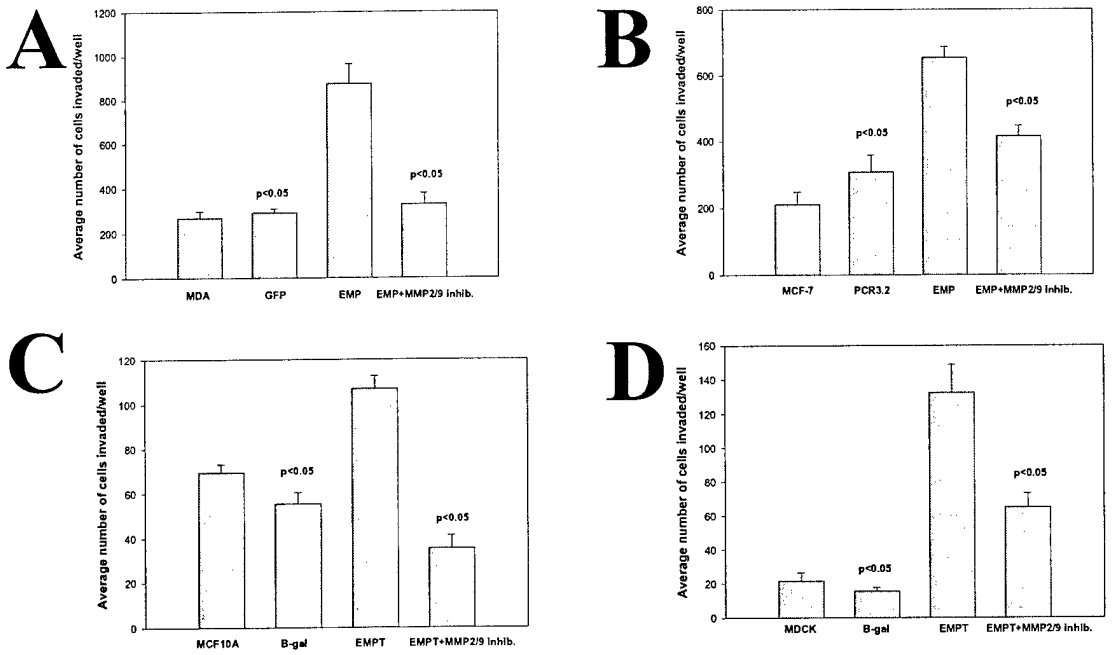

Figure 1. Effect of elevated EMMPRIN expression on cell invasion. EMMPRIN expression was increased via transfection in MDA-MB-436 (A) and MCF-7 (B) cells and via adenoviral infection in MCF10A (C) and MDCK (D) cells. The cells were placed in Matrigel invasion chambers and allowed to invade for 24 hours.

We next examined MMP levels in these cultures to see if the same MMPs that are elevated in heterotypic cultures (epithelial-stromal) are elevated in homotypic (epithelial-epithelial) cultures. MDA-MB-436 (Fig. 2A), MCF-7 (Fig. 2B) and MCF10A (Fig.2C) cells with elevated EMMPRIN levels all showed, by gelatin zymography, increased MMP-2 production over controls. By Western analysis using whole cell lysate we see an increase in MT1-MMP levels in MDA-MB-436 (Fig. 3A), MCF-7 (Fig. 3A) and MCF10A (Fig. 3B) cells with elevated EMMPRIN levels over controls. It is interesting that MMP-2 and MT1-MMP are the only two MMPs in which we see changes given that MT1-MMP is involved in the activation of MMP-2.

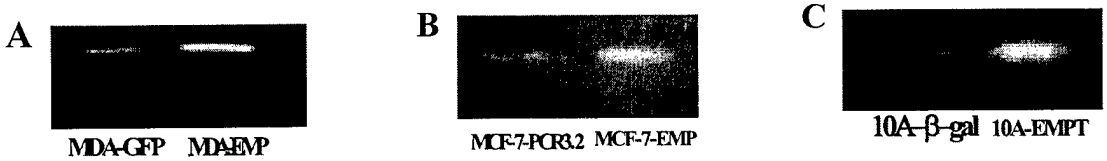

Figure 2. Effect of elevated EMMPRIN levels on MMP-2 production. Gelatin zymography of conditioned media from the following cell types: MDA-MB-436 (A), MCF-7 (B) and MCF10A (C). Lane 1 contains media from control transfectants or infectants and lane 2 contains media from those cells with elevated EMMPRIN levels.

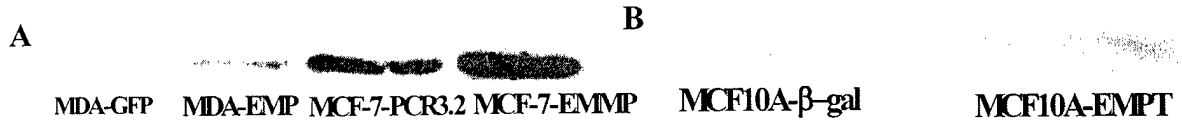

Figure 3. Western analysis of the effect of elevated EMMPRIN expression on induction of MT1-MMP. Lanes contain 15ug of protein from lysates of the following cells: (A) MDA-MB-436, MCF-7 and (B) MCF10A. Lane 1 contains lysate from control transfectants or infectants and lane 2 contains lysate from those cells with elevated EMMPRIN levels.

As mentioned previously, MDA-MB-436 cells transfected with EMMPRIN produce much larger tumors in nude mice than vector-transfected cells. Therefore, we examined whether elevated EMMPRIN levels could enhance anchorage-independent growth *in vitro*, a defining characteristic of transformed cells. MDA-MB-436 (Fig. 4A) and MCF-7 (Fig. 4B) cells with elevated EMMPRIN levels formed 10-fold and 5-fold more colonies respectively over controls in a soft agar assay. MCF 10A and MDCK, both non-malignant cell lines, were incapable of colony formation even with elevated EMMPRIN levels. Therefore, it appears that EMMPRIN is capable of enhancing anchorage-independent growth of an already transformed cell (MDA-MB-436, MCF-7), but is not sufficient to stimulate anchorage-independent growth in a non-transformed cell (MCF10A, MDCK).

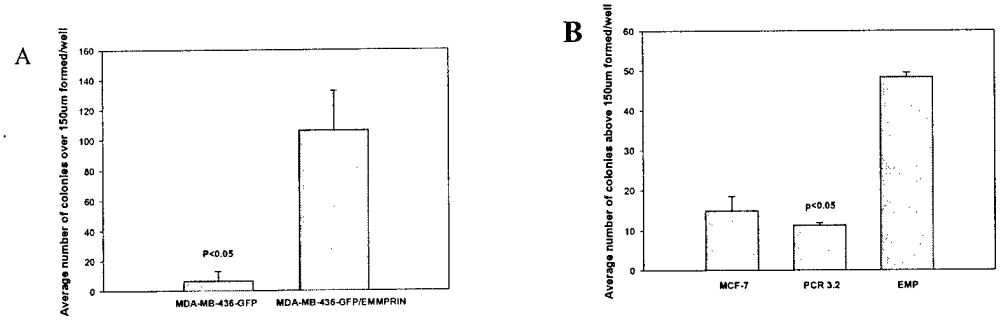

Figure 4. EMMPRIN-enhanced anchorage-independent growth in soft agar. Average number of colonies above 150 um formed per well for (A) MDA-MB-436 (control transfected and EMMPRIN transfected) and (B) MCF-7 cells (untransfected, control transfected and EMMPRIN transfected).

Since anchorage-independent growth often corresponds with elevated levels of hyaluornan (HA), we examined the levels of HA in conditioned media of both a non-malignant (MDCK) and weakly-malignant (MDA-MB-436) cell type by an ELISA-like method. Both MDCK (Fig. 5A) and MDA-MB-436 (Fig. 5B) cells with elevated EMMPRIN levels yielded a 2-fold increase in HA levels over controls.

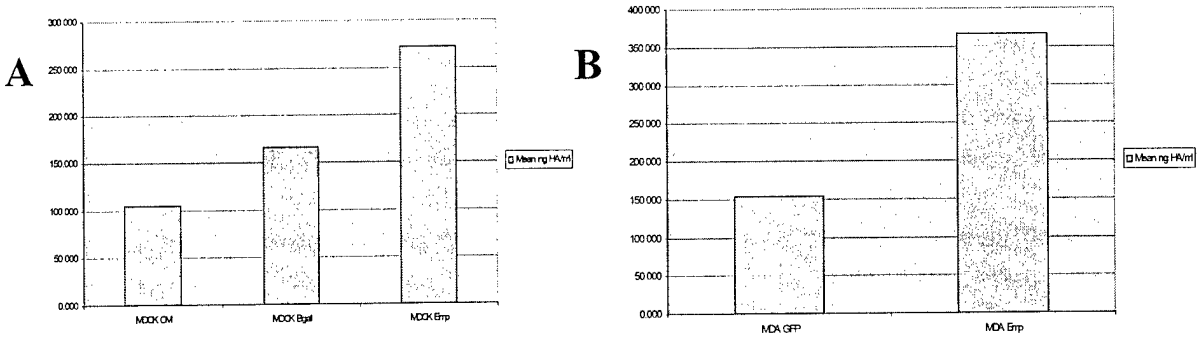

Figure 5. Stimulation of hyaluronan (HA) production by elevated EMMPRIN expression in MDCK canine kidney epithelial cells and MDA-MB-436 mammary carcinoma cells. (A) Elevated EMMPRIN expression by adenoviral infection of MDCK cells leads to elevated HA levels in conditioned media, *(bar 1)* uninfected MDCK, (*bar 2*) MDCK infected with β-gal adenovirus, (*bar 3)* MDCK infected with EMMPRIN adenovirus. (B) Elevated EMMPRIN expression by transfection of MDA-MB-436 cells leads to elevated HA levels in conditioned media, (*bar 1)* MDA-MB-436-GFP, (*bar 2*), MDA-MB-436-GFP/EMMPRIN.

Our laboratory has had success in inhibiting anchorage-independent growth stimulated by enhanced HA production by competing the polymer with HA oligomers. Therefore, we examined whether HA oligomers, fragments of HA polymer, could antagonize EMMPRIN-enhanced anchorage-independent growth. HA oligomer treatment of MDA-MB-436 (Fig. 6A) and MCF-7 (Fig. 6B) cells with elevated EMMPRIN levels resulted in colony formation close to control levels.

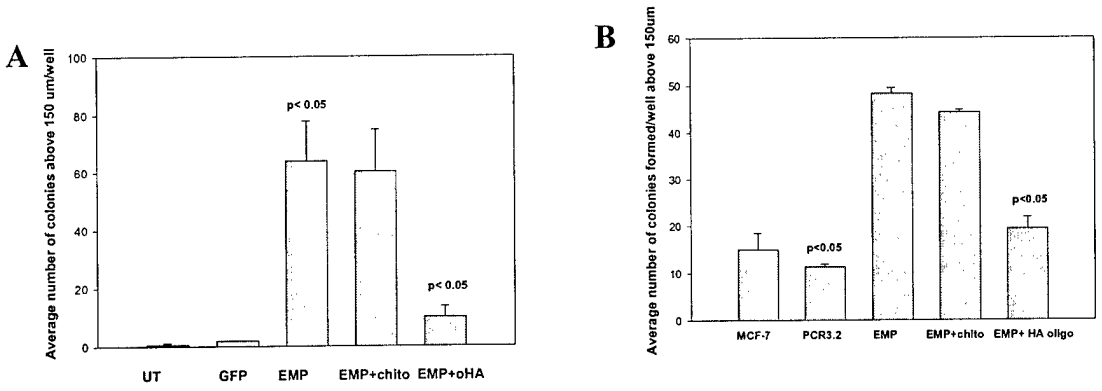

Figure 6. Effect of HA oligomers on EMMPRIN-enhanced anchorage-independent soft agar growth of (A) MDA-MB-436 and (B) MCF-7 cells. Bar1, untransfected; bar 2, control transfected; bar 3, EMMPRIN transfected; bar 4, EMMPRIN transfected + 100ug/ml chito oligomer; and bar 5, EMMPRIN transfected + 100ug/ml HA oligomer.

Task 3: To demonstrate endothelial cell invasion and tubule formation induced by EMMPRIN, or an augmentation of VEGF-induced endothelial cell invasion and tubule formation by EMMPRIN.

One of the systems commonly used to mimic aspects of angiogenesis involves culturing of endothelial cells on 3-dimensional gels of type I collagen or Matrigel. Under appropriate stimulus, the endothelial cells invade the gel and form capillary-like, tubular structures. In one such study (1), human umbilical vein or human dermal microvascular endothelial cells were seeded on collagen gels and treated with a phorbol ester (PMA). With both cell types, PMA treatment induced invasion of the gels and formation of vessel-like structures within the gels, along with increased levels of MMP-1 and gelatinase A. Inhibition of MMP activity especially MMP-1, prevented the induction of endothelial cell invasion and morphogenesis by PMA. These experiments imply that stimulation of MMP activity is necessary, and possibly sufficient, for induction of invasion and tubule formation in this system.

We have conducted experiments in which human umbilical vein endothelial cells were cultured in 12-well plates ($5X10^{5}$) on a type I collagen gel and treated with either bFGF, a known angiogenic agent, or purified EMMPRIN, a known MMP stimulator. Untreated cells maintain a cobblestone-like pattern, whereas both bFGF- and EMMPRIN-treated endothelial

cells form capillary-like tubules, as visualized by phase constrast microscopy. Tubule formation is such, that treating cells with 1ug of purified EMMPRIN yields visual results comparable to those obtained with 5ng of bFGF. We believe that the high amount of EMMPRIN needed is due to inactivation of most of the protein during purification.

Due the the constraints in purifying EMMPRIN from LX-1 cells that were mentioned above in Task 2, we utilized an adenoviral approach. The human umbilical vein endothelial cells were cultured in 12-well plates on type I collagen. Rather than adding exogenous EMMPRIN, we infected the cells with a recombinant, full-length EMMPRIN adenovirus, enabling the cells to make EMMPRIN themselves. By the same principle mentioned previously, we expected the EMMPRIN on the surface of a given endothelial cell to interact with its putative receptor on a neighboring endothelial cell, triggering a series of signaling events that will result in tubule formation, presumably by MMP stimulation. These cultures were assayed by phase contrast microscopy, Western analysis and ELISA. Infection with the recombinant, full-length EMMPRIN adenovirus failed to stimulate capillary-like tubule formation by HUVECs.

Key Research Accomplishments

- Construction of EMMPRIN antisense cDNAs and ribozymes.
- Stable transfection of TA3/ST murine breast carcinoma cells and MDA-MB-231 human breast carcinoma cells with the previously mentioned constructs.
- EMMPRIN-stimulated capillary-like tubule formation by human umbilical vein endothelial cells on type I collagen.
- EMMPRIN-dependent stimulation of MDA-MB-436, MCF-7, MCF10A and MDCK invasion in Matrigel invasion chamber assays by transfection and infection with a recombinant, full-length EMMPRIN adenovirus.
- EMMPRIN-enhanced anchorage-independent growth of MDA-MB-436 and MCF-7 cells in a soft agar assay.
- EMMPRIN-stimulated MMP-2 production by MDA-MB-436, MCF-7 and MCF10A cells.
- EMMPRIN-stimulated MT1-MMP production by MDA-MB-436, MCF-7 and MCF10A cells.
- EMMPRIN-stimulated production of hyaluronan by MDA-MB-436 and MDCK cells.
- HA oligomer inhibition of EMMPRIN-enhanced anchorage-independent growth of MDA-MB-436 and MCF-7 cells.

Reportable Outcomes

- Ph.D. in Cell, Molecular and Developmental Biology-anticipated award date: December 2002

- Manuscript in preparation: Effects of Increased Expression of a Matrix Metalloproteinase Inducer, emmprin, on Tumor Progression.

- Abstract at Era of Hope meeting (Sept. 2002): Effects of Increased Expression of a Matrix Metalloproteinase Inducer, emmprin, on Tumor Progression.

Conclusions

One of the crucial steps in tumor progression is modification of the pericellular matrix surrounding tumor cells. A major class of proteases that mediates the proteolytic aspect of this process is the matrix metalloproteinases (MMPs). The cell surface glycoprotein, EMMPRIN, enriched on the surface of tumor cells, stimulates production of several MMPs by stromal cells and by the tumor cells themselves, implying that emmprin is an important regulator of MMP production during tumorigenesis *in vivo*, making it a potentially crucial component of tumor progression.

We examined whether increased expression of EMMPRIN in weakly-malignant MDA-MB-436 and MCF-7 mammary carcinoma cells and in non-malignant MCF10A mammary epithelial cells and MDCK canine kidney epithelial cells induces anchorage-independent growth and invasion, hallmark characteristics of the malignant phenotype. Using a soft agar assay to assess anchorage-independent growth, increased expression of EMMPRIN stimulated a 10-fold increase in the number of colonies formed by MDA-MB-436 cells. When assessed by invasion of reconstituted basement membranes,we found that EMMPRIN enhanced invasiveness 2 to 9-fold in MDA-MB-436, MCF-7, MCF10A and MDCK cells.

Next, we examined changes in MMP production. Increased expression of EMMPRIN in MDA-MB-436 and MCF-7 cells yielded a 3-fold increase in MMP2 assayed by gelatin zymography. By Western analysis we saw a 3-fold increase in MT1-MMP, an enzyme involved in the activation of MMP2 and in tumor cell invasion.

Hyaluronan, a component of tumor cell pericellular matrix has also been shown to promote tumor progression. Using a competitive ELISA method, we found that increased expression of EMMPRIN in MDA-MB-436 and MDCK cells gave rise to hyaluronan levels 2.5-fold greater than controls. Using hyaluronan oligomers to compete with endogenous hyaluronan polymer, we reversed EMMPRIN-enhanced colony formation of MDA-MB-436 cells to near control levels.

These data imply that EMMPRIN, a cell surface glycoprotein enriched on the surface of tumor cells, promotes tumor progression, most likely through stimulation of hyaluronan and MMP synthesis. Therefore, antagonizing EMMPRIN may be a useful therapeutic avenue towards slowing tumor

References

1. Folkman, J. (1995). Angiogenesis in cancer, vascular, rheumatoid and other disease. Nature Med. **1:** 27-31.

2. Fisher, C., Gilberston-Beadling, S., Powers, E.A., Petzold, G., Poorman, R., and Mitchell, M.A. (1994). Interstitial collagenase is required for angiogenesis in vitro. Develop. Biol. **162:** 499-510.

3. Polette, M. and Bierembaut, P. (1996). Matrix metalloproteinases in breast cancer. Breast J. **2:** 209-220.

4. Heppner, K.J., Matrisian, L.M., Jensen, R.A., and Rodgers, W.H. (1996). Expression of most matrix metalloproteinase family members in breast cancer represents tumor-induced host response. Amer. J. Pathol. **149:** 273-282.

5. Caudroy, S., Polette, M., Nawrocki-Rabi, B., Toole, B.P., Zucker, S. and Birembaut, P. EMMPRIN-induced invasiveness of a human mammary tumor cell line is associated with enhancement of matrix metalloproteinase-2 expression and activity. (Submitted for review)